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Re:	Application of:	Simon Yau Leung CHING
	Serial No.:	To Be Assigned
	International Appl. No.:	PCT/AU2003/001324
	Filed:	Herewith
	For:	**A METHOD FOR MEASURING ANTIOXIDANT STATUS**

LETTER RE: PRIORITY

Sir:

Applicant hereby claims priority of Australian Patent Application No. 2002951886 filed October 8, 2002 through International Application Serial No. PCT/AU2003/001324 filed October 8, 2003.

Respectfully submitted,

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PRIORITY DOCUMENT

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Patent Office
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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002951886 for a patent by THE WESTERN AUSTRALIAN CENTRE FOR PATHOLOGY AND MEDICAL RESEARCH as filed on 08 October 2002.

WITNESS my hand this
Twenty-second day of October 2003

J Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

P/00/009 28/5/91
Regulation 3.2

ORIGINAL

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "A Method for Measuring Antioxidant Status"

The invention is described in the following statement:

"A Method For Measuring Antioxidant Status"

Field of the Invention

The present invention relates to a method for determining the antioxidant status and/or oxidative stress of inorganic and organic matter and kits for measurement thereof.

Background Art

Oxidative stress is a general term often used to describe the positive bias imbalance of free radicals and reactive oxygen species (ROS) in a system under stress. Oxidative stress is closely associated with antioxidants, which act as a counter balance. There is a large amount of literature available showing that antioxidants, oxidative stress, free radicals and ROS are related to many diseases. Diseases such as, aging, atherosclerosis, dementia, autoimmune diseases, and carcinogenesis, may be retarded or prevented by reducing the level of oxidative stress.

The association between diseases, oxidative stress and antioxidants as a whole remains unclear, mostly due to an inability to adequately measure antioxidant status. A question commonly asked is how many and in what amount of antioxidants does one need to reduce oxidative stress and hence reduce the incidence of preventable diseases.

The exact source of free radicals and ROS is unknown. It is suggested that biological processes such as glycolysis, the citric acid cycle, and mitochondria oxidative phosphorylation are some of the oxidative processes, which form by-products such as HOCl, H_2O_2, $HO^{\bullet}$ and $O_2^{\bullet}$, and therefore contribute to oxidative stress. Monocytes, macrophages, and eosinophils, sensitised following activation, and neutrophils all generate free radicals. Endogenous nitric oxide, a free radical, has an important role as a mediator of vascular dilation, neural

transmission, defence against micro-organisms and inhibition of platelet adhesion.

Extracellular free radicals may be formed when cells encounter pollutants, nitrogen oxides, toxic gases, herbicides, drugs and poisons. Stress such as vigorous exercise, heat shock, trauma, ultrasound, hyperoxia, radiation and many diseases release free radicals such as H_2O_2, HO• and $O_2^{\bullet-}$ from cells. Regardless of modes of formation, by-products such as HOCl, H_2O_2, HO•, $O_2^{\bullet-}$ and NO are collectively labelled as free radicals and ROS.

Whereas intracellular free radicals and ROS may be essentially part of life, extracellular free radicals and ROS are usually formed due to environmental factors, diseases and life style such as smoking. Under oxidative stress, excess free radicals and ROS are produced by intracellular and/or extracellular means which may damage bio-molecules directly or propagate free radical chain reaction.

Free radicals and ROS are unstable, reactive and have very short half-life. Scientists have known for a long time that living organisms have different mechanisms to deal with free radicals and ROS. Antioxidants are a group of compounds used by living organisms to neutralize free radicals and ROS. The term "antioxidant status" was first used to describe the ability of a system to deal with free radicals and ROS collectively. However, so far scientists are still struggling to show clinically the beneficial protective effect of antioxidants. A number of big trials such as the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBCCPS), the Beta-Carotene and Retinol Efficacy Trial (CARET) and the Women's Health Study (WHS) cannot unequivocally state the beneficial effect of individual antioxidant against diseases.

Intracellular antioxidants consist of, for example, enzymes, proteins, peptides, and compounds such as ubiquinone, retinol and tocopherol. When the intracellular antioxidant level is low, free radicals and ROS activate oxidant-sensitive transcription factors, such as nuclear factor-$\kappa\beta$ and adaptor protein, which binds to antioxidant response element (ARE) on the loci with oxidative

responsive genes. This leads to synthesis of cell survival proteins such as antiapoptotic proteins Bfl-1 and Bcl-X_L, and antioxidant enzymes such as manganese superoxide dismutase that protect mitochondria. Increase in level of other protective enzymes and proteins such as catalase, glutathione peroxidase, glutathione reductase, glutathione-s-transferase, metallothionein, CuZn-SOD, Mn-SOD, and lipoylated-proteins have also been reported. Damage to DNA, activation of poly-ADP-ribose transferase (PADPRT) and apoptosis proceed when intracellular antioxidant level is not restored.

Extracellular antioxidants form a very diverse group of compounds and are influenced by diet and physiological states. Increased consumption of fruits, vegetable and vitamin supplements are related to an improvement in antioxidant status. Proteins such as extracellular superoxide dismutase (SOD), ceruloplasmain, ferritin, lactoferrin, transferrin, haemopexin, haptoglobin and albumin; biomolecules such as bilirubin, carotenoids (β-carotene, lycopene etc.), flavonoids (quercetin, rutin, catechin etc.), urates, ubiquinol, thiols (R-SH), vitamins A, C, and E; trace elements, such as selenium and zinc are all independent variables of antioxidant status. Low level of extracellular antioxidant may allow free radicals and ROS to react with lipid, protein and carbohydrate biomolecules on cell membrane as well as hormones and enzymes. For example, excess free radicals and ROS promote lipid peroxidation, which in turn may cause cell necrosis.

There are many different ways of measuring oxidative stress and antioxidants status including: (i) measuring free radicals and ROS directly, (ii) measuring oxidative stress by-products, (iii) measuring a combination of antioxidants, and (iv) measuring total antioxidant status.

Firstly, direct measurement of free radicals and ROS is extremely difficult because of their short half-life, instability and low concentration. For instance, HO• has a half-life of about 10^{-9} seconds and exists in the picomolar range. Most important of all the location at the time of formation of free radicals and ROS

inside the body is unpredictable. For these reasons, methods to measure free radicals and ROS directly is impractical.

A second method for measuring antioxidant status is the measurement of oxidative by-products. Reagents used to measure some of the oxidative by-products include: 2,4-dinitrophenylhydrazine, fluorescein thiosemicarbazide and fluorescein amine/cyanoborohydride. A method to measure protein carbonyls in plasma of oxidized LDL by ELISA has also been reported. These methods require specialised expertise, are time consuming and costly and hence not widely used. In addition, the compounds being measured do not necessarily reflect the immediate picture of oxidative stress and antioxidant status.

Further, the results are subjected to misinterpretation caused by excessive non-specific production of these by-products. Alternative factors, other than systemic oxidative processes, may increase lipid peroxidation by-products such as oxidized-LDL, lipofuscin-like compounds, conjugated dienes, malondialdehyde, hydroxyalkenals, and F_2-Isoprostanes. Even apparently healthy subjects show wide variation in levels of oxidative by-products in body fluids.

A third method for determining antioxidant status is to measure a combination of antioxidants. However, this approach also has its drawbacks - the major criticism being in the choice of antioxidants measured. It is plain to see the unlimited permutation possible in choosing the combination of antioxidants to measure. There are many methods available to measure intracellular and extracellular antioxidants. Methods for intracellular antioxidant are available commercially to measure enzymes such as catalase, glutathione peroxidase and superoxide dismutase, and RBC glutathione after derivatization followed by HPLC.

Total intracellular antioxidant status measurement may be difficult to perform because most of these compounds are proteins and enzymes. When it comes to extracellular antioxidant, there are a huge varieties of compounds, which may be grouped under categories such as vitamins, trace elements, thiols, enzymes, natural products, and biological molecules.

It is indeed not practical to measure each and every one of the antioxidants *in vivo*. It is also now widely hypothesized that the major factor influencing oxidative stress is the overall *antioxidant status* of the system, which prevents diseases by eliminating free radicals and ROS. Therefore, it is essential to have a method capable of measuring collectively the extracellular antioxidant status.

Finally, there are methods for measuring antioxidant status which are based on the inhibition of generated free radicals reaching the target indicator molecules, by antioxidants. The common feature for inhibition assays is to generate a free radical to react with a target molecule, thereby generating an endpoint that can be observed and quantified. Addition of antioxidants inhibits the development of this endpoint. A good example of this is the Trolox equivalent antioxidant capacity (TEAC) method based on the quenching of the absorbance of the radical cation formed by the reaction of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS) with metmyoglobin and H_2O_2.

The inhibition of the free radical action by an antioxidant in this assay depends on lag-phase and degree of inhibition. Lag-phase in this assay is defined as the time where there is a total inhibition of peroxyl radical action by antioxidant. Some compounds show good lag-phase such as Trolox and uric acid but others like albumin show no lag phase unless at very high concentration.

However, there are three major problems relating to the use of lag-phase measurement in for example, body fluids. Firstly, use of lag-phase measurement assumes linear reaction rate between the peroxyl radical generator and target molecule. Secondly, body fluid with high protein content and some other compounds do not produce lag-phase at low concentrations. Thirdly, contribution to the antioxidant activity by plasma proteins such as albumin will not be included in the final results.

Another method, based on inhibition, which aims to eliminate the shortcomings of the TEAC method, has been developed. However, it also has its associated problems. This method combines both inhibition percentage and lag-phase of the free radical action by antioxidant into a single result and was named the

oxygen radical absorbance (ORAC) assay. Cao GH et al., (1995) [*Clin. Chem.*; 41/12: 1738-44] modified the method by Glazer AN (1990) [Methods Enzymol; 186:161-168], improving it by using an area under the curve technique for quantification.

Again, there are several major draw backs with the ORAC assay. Firstly, there are chemical inconsistency and instability problems associated with the fluorescent probe molecule phycoerythrin. Phycoerythrin varies from batch to batch since it is obtained on the basis of protein content with approximately 30% protein (Lowry) and the rest primarily sucrose, dithioerythritol and sodium azide.

Secondly, the operational temperature of the ORAC assay is at 37°C which is in direct conflict with the stability of phycoerythrin and the free radical reagent, 2,2'-azobis(2-amidinopropane) dihydrochloride in solution, both being unstable at 37°C. Phycoerythrin also suffers from photosensitivity and is highly toxic.

Thirdly, this method relies on a one-point calibration rather than the use of a more accurate standard curve. The ORAC method has been modified to replace phycoerythrin with fluorescein as the fluorescent probe. However, this assay also has to be performed at 37°C.

There are other methods in the literature recently trying different indicator and radical generating reagents, such as the method using 4,4-difluoro-4-bora-3a,4a diaza-s-indacene fluorophore with 2,2'-azobis-2,4-dimethyl valeronitrile. The operational temperature of this method is also at 41°C using a Shimadzu RF 1501 spectrofluorophotometer in a 1 cm quartz cuvette requiring stirring for 60 minutes. This method is very time consuming and cumbersome. Large-scale studies with many specimens are therefore not possible with this method.

A method known as the FRAP assay (Ferric Reducing/Antioxidant Power) measures, at low pH, reduction of a ferric tripyridyltriazine complex to the ferrous form, which has a blue colour measurable at a wavelength of 593 nm. This method has been criticised for failing to distinguish between a biological antioxidant and a chemical reductant. In addition, the FRAP assay does not

measure sulphur containing compounds such as glutathione. Also, some antioxidants may not be able to reduce Fe^{3+} *in vivo* and Fe^{2+} is itself a pro-oxidant *in vivo* converting H_2O_2 to HO•.

As discussed above, there are a number of methods available in the literature for determining oxidative stress and antioxidant status. However, none of these methods are totally satisfactory for the reasons mentioned above. Therefore, there is a need to provide a method to overcome at least some of the problems associated with the methods known in the prior art.

Summary of the Invention

The method of measuring oxidative status and/or oxidative stress in a sample comprises the steps of:

(a) contacting the sample with at least a free radical generating substance and a fluorescing porphyrin compound,

(b) determining the antioxidant status and/or oxidative stress of the sample by measuring the resultant fluorescence of the mixture in step (a), and comparing the fluorescence to a standard.

A kit for determining the antioxidant status and/or oxidative stress of a sample, comprising a free radical generating substance, a fluorescing porphyrin compound and instructions for their use.

Brief Description of the Drawings

FIGURE 1 A graphical representation of the fluorescence intensity for Trolox standard at varying concentrations plotted over time.

FIGURE 2 A standard curve generated by measuring the fluorescence intensity plotted over time for varying concentrations of a standard antioxidant, Trolox.

FIGURE 3 A graphical representation of the fluorescence intensity for a variety of coffee powders, chocolate milk powder flavourings and tea plotted over time

FIGURE 4 A graphical representation of the fluorescence intensity for a variety of teas plotted over time

FIGURE 5 A graphical representation of the fluorescence intensity for a variety of wines plotted over time

FIGURE 6 A graphical representation of the fluorescence intensity for a variety human serum samples plotted over time

Disclosure of the Invention

General

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively, and any and all combinations or any two or more of the steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

The entire disclosures of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference. No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

As used herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Description

The present invention provides a simple and reliable method for the measurement of the antioxidant status and/or oxidative stress of inorganic and organic matter. In particular, the method of the present invention incorporates a new type of fluorescent probe for the measurement of extracellular or intracellular antioxidant status. This method has been named the antioxidant inhibition of radical assay or AIOR assay.

The method of the present invention is capable of measuring intracellular antioxidant status, such as extracts of cell culture and cell homogenates. However, the intracellular antioxidant status may vary depending on the adequacy of the cellular response mechanisms present. In addition, many of the intracellular antioxidants are enzymes, which are not measured by this method. In one preferred embodiment, the extracellular antioxidant status is measured.

The method of measuring oxidative status and/or oxidative stress in a sample comprises the steps of:

(a) contacting the sample with at least a free radical generating substance and a fluorescing porphyrin compound,

(b) determining the antioxidant status and/or oxidative stress of the sample by measuring the resultant fluorescence of the mixture in step (a), and comparing the fluorescence to a standard.

The method of the present invention generates an endpoint with a free radical generating reagent that can be quantified. Briefly, the presence of antioxidants in a sample inhibits the breakdown of the fluorescent porphyrin by the free radical generating reagent. The resulting change in fluorescence is an indication of the amount of antioxidant present in a sample. That is, the more antioxidant present in a sample, the slower the rate of degradation of the porphyrin probe.

Porphyrins form a diverse group of compounds. By way of example only, certain substituted porphyrin structures that may be used include the trivially named uroporphyrin I, uroporphyrin II, uroporphyrin III, and uroporphyrin IV; coproporphyrin I, coproporphyrin II, coproporphyrin III, and coproporphyrin IV; MS-tetraphenylporphyrin; deuteroporphyrin IX; hematoporphyrin IX; mesoporphyrin; protoporphyrin IX; dihydrochloride and methyl ester derivatives of all the above mentioned porphyrins.

In a highly preferred embodiment, the probe is of a uroporphyrin structure. More preferably, the probe is uroporphyrin I dihydrochloride.

The concentration of porphyrin used in the method of the present invention varies depending on a number of factors. One factor effecting the concentration of the porphyrin solution used in the method of the present invention is the absorption coefficient (mM) of the particular porphyrin used. That is, if the absorption coefficient for the porphyrin is regarded as high, the concentration of the porphyrin solution will be lower than porphyrins with a lower absorption coefficient.

A further factor effecting the concentration of porphyrin used is the type of equipment used to measure the resulting fluorescence signal. For example, a sensitive spectrofluorometer may require a lower concentration of porphyrin solution than a less sensitive model.

Another factor affecting the concentration of porphyrin solution used in the method of the present invention is the volume of the sample container required by the equipment used to measure the fluorescence signal. For example, larger sample containers, such as 1 cm cuvettes, require a relatively low concentration of porphyrin solution. In one embodiment, a concentration of 90 nmol/L of uroporphyrin I dihydrochloride was sufficient to generate a measurable fluorescence signal. In a further embodiment, a 96 well plate was used in the method of the present invention, and a concentration range of about 180 nmol/L to about 300 nmol/L was used. A higher concentration range may be required for plates with 384 wells.

A person skilled in the art would be able to determine the appropriate concentration of porphyrin solution required for use of the present invention with a variety of different types of equipment capable of measuring fluorescent signal.

Porphyrins are soluble in a range of solvents. For example, porphyrins are soluble in water, chloroform, pyridine, cyclohexanone, benzene, dioxane and p-dioxane. Less ionic porphyrins (e.g. esterified derivatives) tend to be more soluble in are soluble in dioxane or chloroform.

For example, dihydrochloride derivatives of uroporphyrin I, II, III, IV, and coproporphyrin I, II, III and IV, dihydrochloride derivatives of hematoporphyrin IX, uroporphyrin I, II, III, IV, and coproporphyrin I, II, III and IV, mesoporphyrin and protoporphyrin IX are soluble in aqueous solution.

Further, methyl ester derivatives of uroporphyrin I, II, III and IV and coproporphyrin I, II, III and IV, deuteroporphyrin IX dimethyl ester, hematoporphyrin IX dimethyl ester, mesoporphyrin dimethyl ester and protoporphyrin IX dimethyl ester are soluble in chloroform.

In addition, uroporphyrin II octamethyl ester is soluble in pyridine. Uroporphyrin III is soluble in cyclohexanone. MS-tetraphenylporphyrin is soluble in benzene. Coproporphyrin I Tetramethyl ester, Coproporphyrin II Tetramethyl ester derivatives of coproporphyrin I, II, III and IV and deuteroporphyrin IX dimethyl ester are soluble in dioxane. Finally, mesoporphyrin dimethyl ester and protoporphyrin IX dimethyl ester are soluble in p-dioxane.

For measuring the antioxidant status using the method of the present invention in biological systems, the porphyrins are preferably soluble in an aqueous solution. Some porphyrins which are more soluble in organic solvents, such as dioxane, are more suitable for measuring hydrophobic antioxidants, such as Vitamin A and some lipids.

The porphyrins used in the method of the present invention are relatively stable at room temperature. That is, the probe(s) used in the present method are less temperature dependent than the probes used in other fluorescent methods. This allows for the reaction of the present invention to be carried out at room temperature. Specialised equipment for heating and problems associated with diffusion of heat and the effects of temperature on reagent reactivity and stability are therefore not important considerations to be taken into account.

Any free radical generating reagent known to those skilled in the art may be used. By way of example, free radical generating reagents include peroxidase/H_2O_2, horseradish peroxidase/ H_2O_2 and Cu^{2+}/ H_2O_2. In a highly preferred embodiment, the free radical generating reagent is 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH).

Other compounds may be incorporated into the method for the purpose of enhancing the reproducibility and accuracy of measuring the oxidative status and/or oxidative stress of various types of inorganic and organic matter.

For example, to measure the antioxidant status of a sample containing both hydrophobic and hydrophilic antioxidants, it is often necessary to include for example, surfactants, emulsifiers or solubilisers. In a preferred embodiment, the

surfactant belongs to the polyoxyethylene alcohols group. Other names for this group of compounds include polyethylene glycol fatty alcohol ethers; ethoxylated fatty alcohols; macrogol fatty alcohol ethers; alcohol ethoxylates; polyoxyethylene alcohol ethers or polyoxyethylene ethers.

The above compounds are nonionic surfactants prepared by ethoxylation of fatty alcohols with ethylene oxide. Many of these compounds are known by their Trademark. For example, some commercially available series of compounds include: Alfonic, Bio Soft EA, Brij 35™, Dehydol, Emulphogene, Ethosperse, Eumulgin, Ethoxyol, Lipal, Lipocol, Macol, Polychol, Siponic, Trycol, Volpo.

In a highly preferred embodiment, the surfactant is polyoxyethylene ethers, such as Brij 35™.

The method of the present invention is adaptable to high throughout methods. This type of sample analysis may be achieved for several reasons. Firstly, the stability of the fluorescent probe at room temperature allows for a large number of samples to be measured at one time. For example, the fluorescent probe reagent may be delivered to the wells of a 96-well plate and not be effected by the delay between mixing of reagents and sample and the time of reading.

Secondly, the method of the present invention is suitable for scaling down to a micro-assay technique. Therefore, the volume of sample and reagents required is minimal.

Finally, the reagents used in the method of the present invention are not costly and readily available, thus allows the analysis of many samples.

The degradation of the porphyrin probe is measured using fluorescence spectroscopy. Any type of fluorometer capable of measuring fluorescence may be used. For example, any spectrofluorophotometer capable of holding a cuvette or a 96 well plate may be used. In a highly preferred embodiment, the Varian Cary Eclipse fluorescence spectrophotometer, which is capable of reading 96 well-plates, may be used to measure the change in fluorescence.

The Varian Cary Eclipse fluorescence spectrophotometer monitors each of the 96-wellplate spectrums with individual window in real time and also provides automatic data analysis and processing.

With the Varian Cary Eclipse fluorescence spectrophotometer, there is the option for expansion to higher format which enables large throughput of samples. This method takes into account the lag-phase and degree of inhibition of antioxidants and probe, in the presence of a free radical reagent, at room temperature. The area under the curve is related to antioxidant concentrations, and the standard curve is linear.

The method of the present invention may be used to measure antioxidant status and/or oxidative stress of various inorganic and organic matter. For example, the antioxidant status may be assessed in food items, such as fruit, vegetables, meats, dairy products, cereals, grains, food additives, seafood, traditional herbal medicine, vitamins, preserved fruit and vegetables and natural plant extracts; beverages, such as tea, coffee, chocolate flavoured powders, soft drinks, wine, beer, or fruit juice; and biological compounds, such as thiols and lipids. In addition, the oxidative status and/or oxidative stress of biological fluids, such as blood and urine, may also be assessed using the method of the present invention.

The oxidative status of samples, such as biological fluids may be measured to assess the health risk of an individual. In addition, the oxidative status may be used directly or indirectly in the treatment of diseases such aging, atherosclerosis, dementia, autoimmune diseases, and carcinogenesis. If the oxidative status of an individual is known, it may then be possible to provide suitable treatment, since many diseases may be prevented or retarded by reducing the level of oxidative stress.

Further uses of knowing the oxidative status of inorganic or organic matter include: verifying the antioxidant status in foods, drinks and supplements as claimed by the manufacturer; quality control of antioxidants in substances such as tea, coffee, wines and other food items; accurately measuring the oxidative

status of a drug which may aid in the development of antioxidant drugs for the treatment of disease.

In a second aspect of the invention, there is provided a kit for determining the antioxidant status and/or oxidative stress of a sample, comprising a free radical generating substance, a fluorescing porphyrin compound and instructions for their use.

Best Mode(s) for Carrying Out the Invention

Sample Preparation

Samples tested included various brands of coffee and tea, spices, chocolate powdered milk flavouring (Milo®), fruit tea, red wine, white wine, crushed grape juice and human serum samples.

Samples were diluted with phosphate buffer (75mM, pH 7.0), unless otherwise specified. Serum, wine and crushed grape juice were diluted to 1:6, 1:10 and 1:20 (v/v) respectively before analysis. A standard amount of 1.87 mg per ml water of spices, coffee, cocoa, Milo® and tea were used for extraction and comparison purposes.

Chemicals and Equipment

2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), (±)6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), Brij 35 solution and uroporphyrin I dihydrochloride were obtained from Sigma Aldrich (Castle Hill, NSW, Australia).

All samples were analysed on the Cary Eclipse fluorescence spectrophotometer (Varian Australia Pty Ltd, Mulgrave, Victoria, Australia). Sample analysis was carried out in 96-well round bottom polypropylene microplates with a capacity of 330 µL, and were purchased from Alltech Associates Australia (Baulkham Hills, NSW, Australia). A 1296-001 plateshaker was used(LKB-Wallac (Heidolph, Schwabach, German) to mix the samples with the reagents.

Reagents and Standard

The stock phosphate buffer (0.75 M, pH 7.0) was prepared by mixing 61.1:38.9 (v/v) of a 0.75 M K_2HPO_4 and a 0.75 M NaH_2PO_4 solution. The working phosphate buffer 75 mM, pH 7.0 was prepared by a 1:9 (v/v) dilution of the 0.75 M stock phosphate buffer.

AAPH (583 mM) was prepared by dissolving completely 0.474 g of AAPH in 3 mL of 75 mM, pH 7.0 phosphate buffer. This solution was prepared freshly before each run.

Uroporphyrin stock solution (225 µM) was prepared by dissolving 1.2 mg of uroporphyrin I dihydrochloride in 5.9 mL of phosphate buffer (75mM, pH 7.0). This solution was very stable for many months by storing in the dark at 4°C. A secondary uroporphyrin stock solution for routine use was prepared by a 1:50 (v/v) dilution of the stock uroporphyrin solution with phosphate buffer (75mM, pH 7.0). This secondary stock solution was stable for several months in the dark at 4°C. A working uroporphyrin solution for daily use was prepared by a 1:750 (v/v) dilution of the secondary uroporphyrin solution with phosphate buffer (75mM, pH 7.0).

To enable complete solubility of both hydrophobic and hydrophilic compounds in the sample, a surfactant Brij 35™ was added to the working uroporphyrin solution in the quantity of 0.1% of the reagent.

A stock Trolox standard solution (5.0 mM) was prepared by dissolving Trolox 35 (250 mg) in 200mL of phosphate buffer (75mM, pH 7.0). Working standards of Trolox were prepared by diluting the stock Trolox standard to 0.5, 1.0 and 2.0 mM with the same buffer.

Sample Analysis

Sample/blank/standard (1 µL), and working uroporphyrin solution with Brij™ (136 µL) were added to wells of a 96-well micro-plate. The working uroporphyrin

solution with Brij™ was added first into the well, followed by one of either the blank, standards or diluted samples. The contents of each well was mixed using a plateshaker for 1 minute. Freshly prepared AAPH solution (22 µL) was then added. The contents of the wells were then mixed again for exactly 2 minutes before acquisition of data with the Varian Cary Eclipse fluorescence spectrophotometer.

Fluorescence monitoring and Data Processing:

The Varian Cary Eclipse fluorescence spectrophotometer settings were as follows.

Excitation λ = 397 nm; Emission λ = 615 nm.

Excitation Slit = 20 nm; Emission Slit = 10 nm.

Ave Time = 0.2 sec.

Run Time = 170 minutes.

Y (min – max) = 0 – 1000.

Excitation Filter: Auto.

Emission Filter: Open.

PMT: High.

The results of analysis were calculated using the Analyze: Simple calculation; Stage 1; Stop 1 min; Order First; Plot Fit, as defined in the User's manual.

The method takes the free radical action to completion in 170 minutes. The Varian Cary Eclipse fluorescence spectrophotometer has the ability to achieve high throughput of 96 samples in 170 minutes with the 96-wellplate format. Both time and cost of this assay per sample is greatly reduced by this method. This

invention provides both a method and reagent, which can accurately, easily, economically, and quickly analyze antioxidant status.

Results

Uroporphyrin fluoresces in solution at a wavelength of 406 nm in 1 M HCl and has a maximum absorption coefficient (ε_{mM}) of 505. In a 0.5 M HCl solution, uroporphyrin fluoresces at 406 nm with an absorption coefficient (ε_{mM}) of 541. Free radicals and ROS breakdown uroporphyrin thereby resulting in a degradation of the fluorescent intensity over time. However, the presence of antioxidants in the sample or standard solution prevents the degradation of fluorescence. Therefore, a fluorescent intensity maintained over time is an indication of a higher concentration of antioxidants. That is, the longer the fluorescent intensity is maintained the greater the amount of antioxidants present in the sample.

Figure 1 shows the result of the fluorescent intensity of standard solutions of Trolox (0 to 2.0 mM) measured over time. As can be seen from the graph, a standard solution of 2 mM Trolox maintains a fluorescent intensity for a longer time than standard solutions of Trolox at 0 to 1.0 mM.

The results of the above experiment were plotted as fluorescent intensity versus concentration of Trolox, as shown in Figure 2. The result is a linear standard curve from at least 0.5 mM to at least 2.0 mM.

A variety of brands of instant coffee powder, chocolate flavoured powder and tea powder were tested for their antioxidant status. Figure 3 shows the results. From the graph, it can be seen that coffee powders have a higher antioxidant status than chocolate flavoured powders and tea powder.

Similar curves were generated from a variety of different types of teas and brands of teas. Figure 4 shows the comparison of the ability of antioxidants in a range of teas to inhibit the free radical reagent. Figure 5 shows the results of different types of wine at a 1 in 10 and 1 in 20 dilution. The antioxidant status

was also measured in human serum samples (Figure 6). As can be seen from the graphs, the antioxidant status varies between teas, coffee, wines and individuals.

A comparison of the antioxidant status of tea, coffee, chocolate flavoured powders and wine was made with the antioxidant standard, Trolox. The comparison is expressed as a number relative to Trolox (mM) (see Table 1). A higher number indicates a greater antioxidant status. The tea, coffee and chocolate flavoured powder were all diluted to 1.87 mg/ml. The wine was used undiluted. Each sample (n=24) was analysed using the method of the above method and the coefficient of variation measured (CV%).

Name	Trolox Equivalent (mM)	CV(%)
Gibsons traditional full strength tea	3.0	5.0
Twinings traditional afternoon tea	3.5	3.8
Butterfly Brand Fujian China tea	3.7	6.5
Twinings green tea and mint	2.8	4.8
Tetley Emperor's Garden green tea and peppermint	2.7	5.2
Twinings blackcurrant/apple	1.2	10.1
Nescafé Blend 43 coffee	3.5	4.9
Nescafé Gold coffee	3.3	4.3
Nescafé Decaffeinated coffee	3.6	4.6
Nestlé Cocoa	1.3	6.2
Milo	0.4	13.6
Green tea powder Japan	0.2	27.0
Jacob's Creek Chardonnay Vintage 2001	10.4	6.3
Wyndham Bin 444 Cabernet Savignon Vintage 2000	34.6	4.0

Table 1

A similar comparison of the antioxidant status of red and white wine was carried out with Trolox. Each sample was measured 6 times and the coefficient of variation is given in Table 2. It appears from these results that red wine has a greater antioxidant status than white wines.

Name of wine	Trolox Eqivalent (mM)	CV(%)
PEM Cabernet/Merlot	50.1	2.6
PEM Pinot 1	46.9	2.5
MR Cabernet	46.2	2.6

Wyndham Bin 444 Cabernet Sauvingnon (2000)	45.3	2.4
MR Shiraz	38.6	1.9
PEM Pinot 2	34.7	1.9
Jacob's Creek Chardonnay (2001)	24.3	16.6
DUNS Chardonnay	21.9	10.7
MR Chardonnay	16.5	8.0
PEM Chardonnay	15.8	8.6
MR Mix	15.0	11.3

Table 2

Finally, a comparison of the antioxidant status of the serum from human subjects was made with Trolox (mM). In addition, the amount of Bilirubin (µmol/L), uric acid (µmol/L), albumin (g/L) and total protein (g/L) have been compared to the oxidative status of the serum samples. The results of this comparison are presented in Table 3.

Sample	Trolox Equivalent (mM)	Bilirubin (µmol/L)	Uric acid (µmol/L)	Albumin (g/L)	Total protein (g/L)
QC 58-01	9.1	25	280	28	46
QC 58-05	11.3	37	370	33	53
P4201873P	11.7	4	490	39	81
P4201869L	11.9	8	270	41	79
QC 58-16	17.9	68	600	45	72
QC 58-02	18.6	69	600	43	73

Table 3

Dated this EIGHTH day of OCTOBER 2002.

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Patent Attorneys for the Applicant

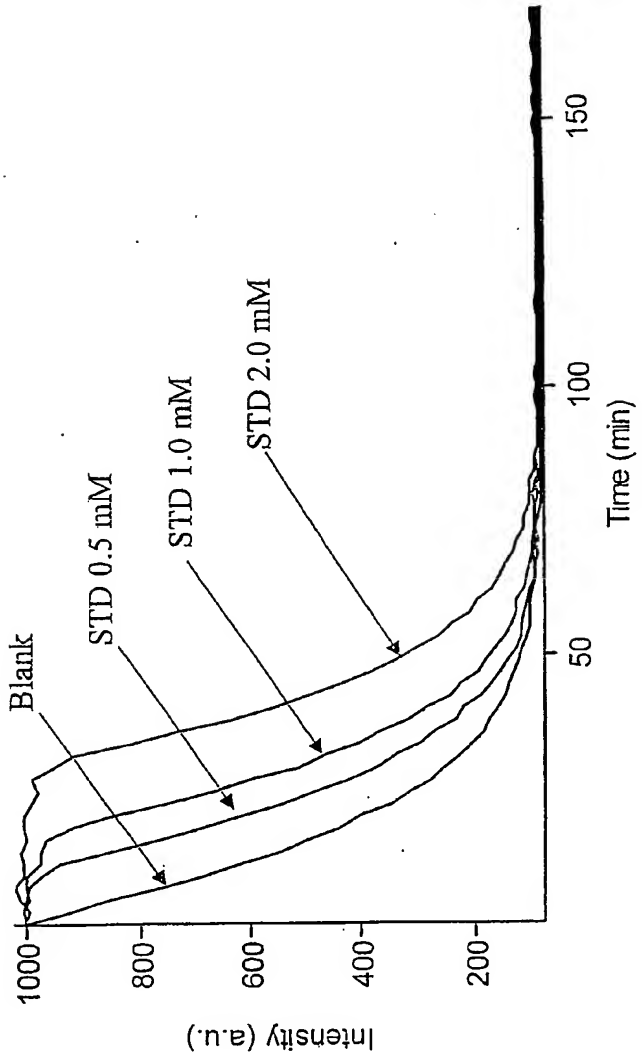
Blank
STD 0.5 mM
STD 1.0 mM
STD 2.0 mM
1000
800
600
400
200
Intensity (a.u.)
50
100
150
Time (min)

Figure 1

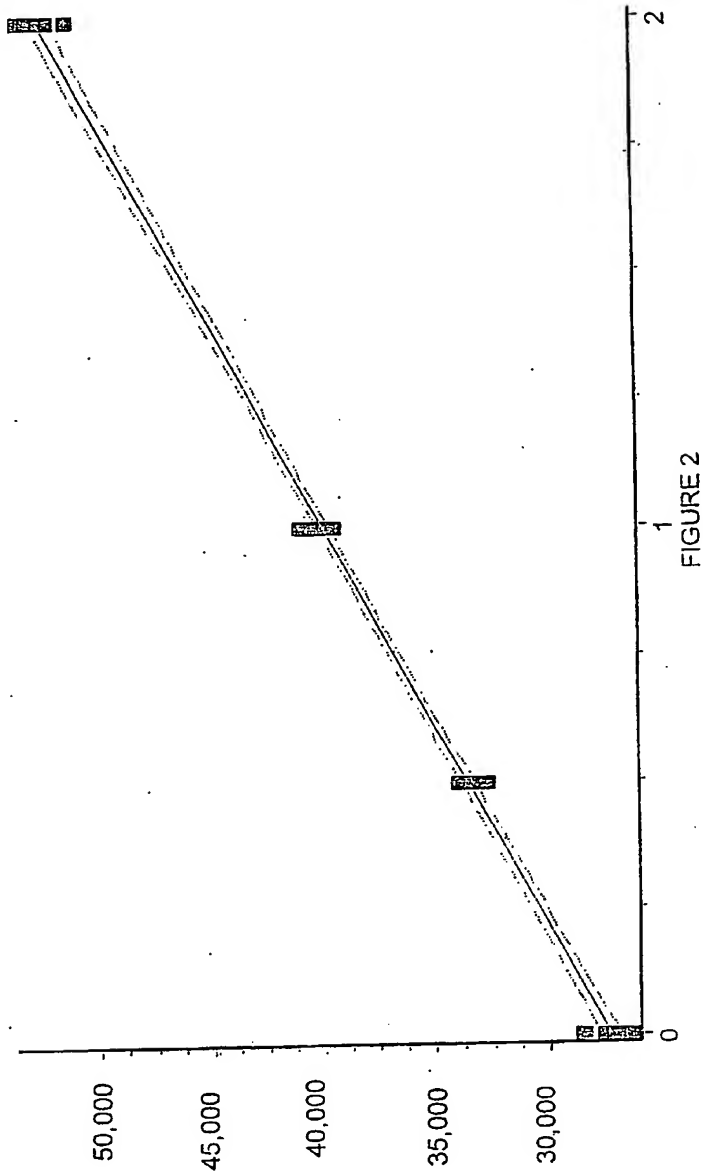

FIGURE 2

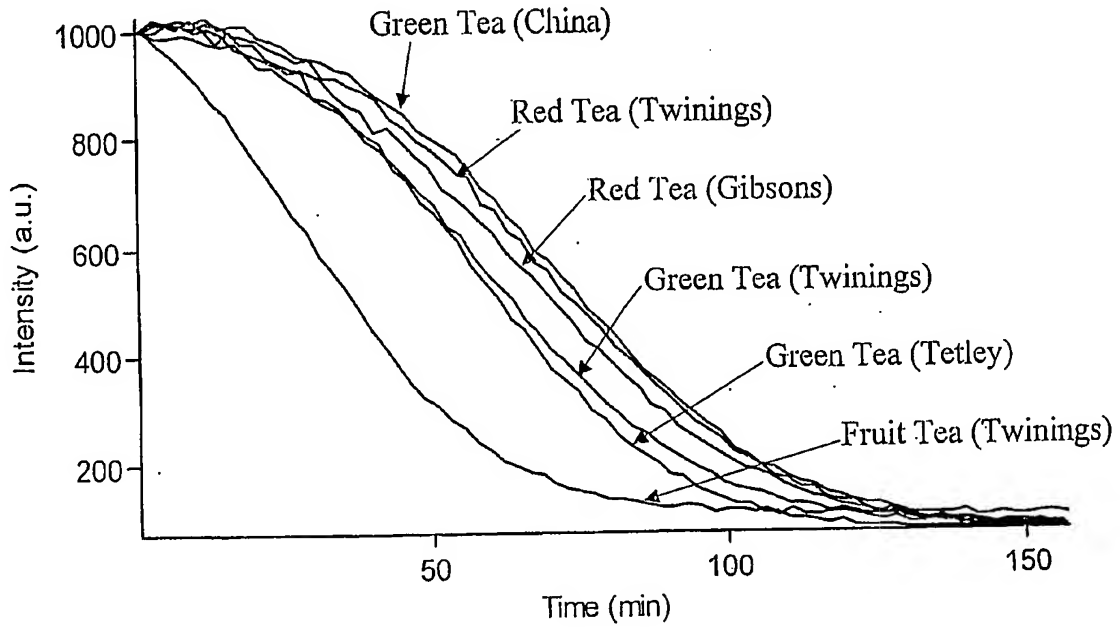
Green Tea (China)
Red Tea (Twinings)
Red Tea (Gibsons)
Green Tea (Twinings)
Green Tea (Tetley)
Fruit Tea (Twinings)
1000
800
600
400
200
Intensity (a.u.)
50
100
150
Time (min)

FIGURE 3

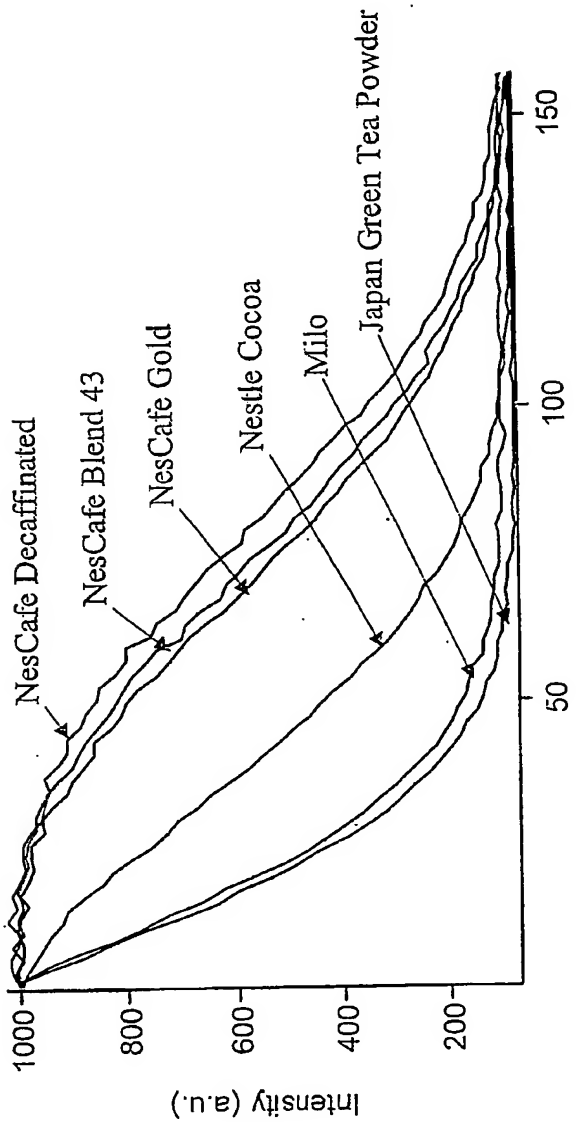
NesCafe Decaffinated
NesCafe Blend 43
NesCafe Gold
Nestle Cocoa
Milo
Japan Green Tea Powder
Intensity (a.u.)
1000
800
600
400
200
50
100
150
Time (min)

FIGURE 4

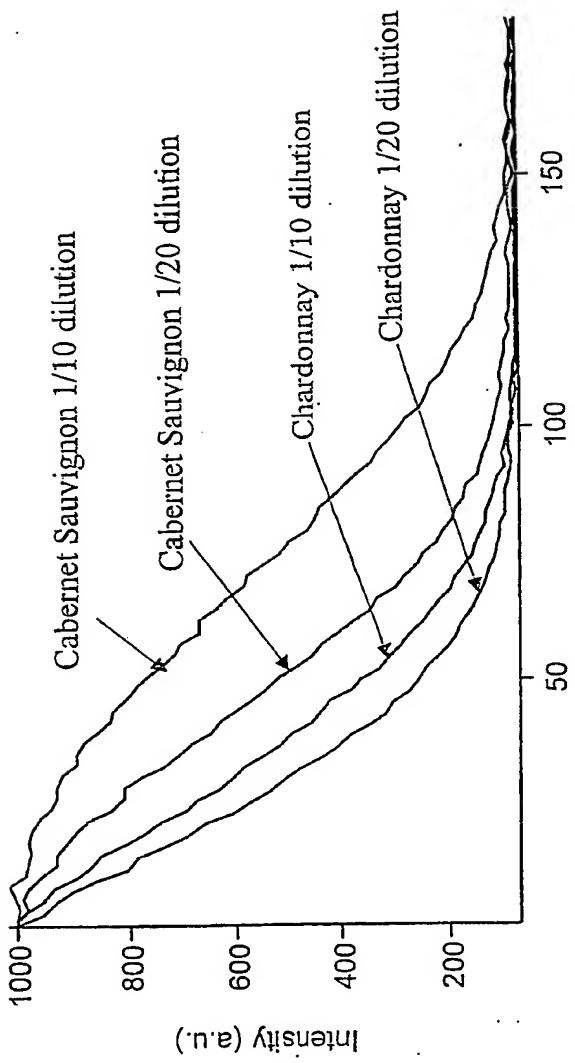
Cabernet Sauvignon 1/10 dilution
Cabernet Sauvignon 1/20 dilution
Chardonnay 1/10 dilution
Chardonnay 1/20 dilution
Intensity (a.u.)
1000
800
600
400
200
50
100
150
Time (min)

FIGURE 5

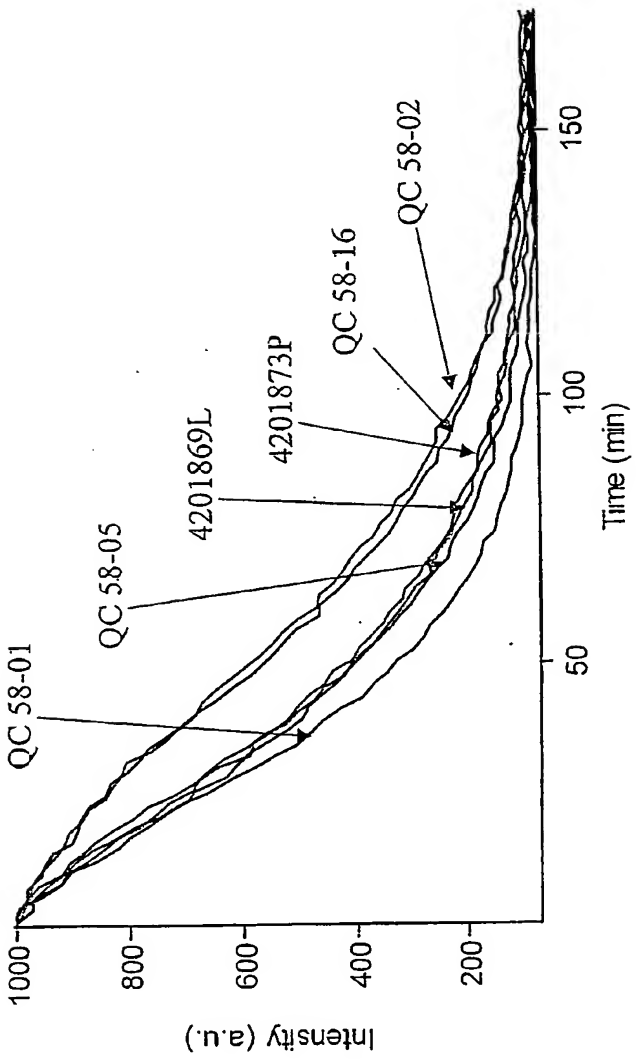
Intensity (a.u.)
1000
800
600
400
200
50
100
150
Time (min)
QC 58-01
QC 58-05
4201869L
4201873P
QC 58-16
QC 58-02

FIGURE 6